An evaluation of two heavier than water internal limiting membrane specific dyes during macular hole surgery

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Abstract

Purpose: To evaluate the staining characteristics, and effect on internal limiting membrane (ILM) histology of two heavier than water ILM specific dyes during macular hole surgery: Acid violet 17 combined with 5% mannitol (AV17-M) and Brilliant Blue G with 4% polyethylene glycol (BBG - P)

Methods: Single centre observational comparative cohort study. The ILM of consecutive patients undergoing surgery for idiopathic macular hole were stained with BBG-P and AV17-M for 10 seconds each. The ILMs were retrieved and examined with electron microscopy. The extent of retinal and vitreous side debris scored. Surgical videos were used to assess the staining contrast effect by measuring the Euclidean distance in the CIELAB colour space between stained and unstained retina after peeling.

Results: 51 consecutive patients were studied with 25 in the AV17-M group and 26 in the BBG-P group. The mean age was 71 years with no significant difference between the groups. The amount of retinal side tissue was greater on the BBG-P stained ILMs compared to the AV17-M stained ILMs (30.2% versus 19.6%, $p<0.001$). There was a difference in the CIELAB colour space separation distance between stained and peeled retina (5.89 versus 3.97, $p=0.01$) in favour of BBG-P. Visual outcomes between the two groups were similar (logMAR visual acuity 0.40 versus 0.38, $p=0.74$)

Conclusion: Both stains were successfully used to peel ILM with comparable outcomes. AV17-M resulted in less retinal debris than BBG-P suggesting an altered and potentially beneficial ILM cleavage plane from the retina but with lowered staining contrast than BBG-PEG.

Key words

Acid Violet 17, Brilliant Blue G, Staining, Internal limiting membrane, Macular hole

Introduction

A variety of vital dyes have been used to facilitate peeling of the internal limiting membrane (ILM) during macular hole surgery. Surgeon choice of dye is influenced by many factors including the potential for retinal toxicity, as well as the specificity and colour contrast of the staining effect. Additionally it is thought that dyes can potentially alter the cleavage plane of where the ILM separates from the retina and also alter the biomechanical properties of the ILM with an increase in stiffness making the tissue easier to peel [1-5]. Indocyanine green was the first dye utilised for ILM peeling but its use has declined with concerns over its toxicity [6]. More recently Brilliant blue G (BBG) has been used by many surgeons and it is now marketed in heavier than water formulations with the addition of 4% polyethylene glycol (BBG-P)(ILM blue, DORC, the Netherlands) or deuterium oxide (Brilliant peel, Fluoron, Germany) [7,8]. The chemically similar aminotriarylmethane dye Acid violet 17 (AV17) has recently been introduced as an alternative to BBG and toxicity studies have shown acceptable profiles in an in vivo rabbit model and in an ex vivo bovine retina model [9, 10]. It is marketed in a heavier than water preparation with 5% mannitol (AV17-M) (Alapurple, Alamedics, Germany). We evaluated the use of AV17-M and compared it to BBG-P in a cohort of patients undergoing macular hole surgery.

Method

A consecutive series of patients undergoing macular hole surgery by one surgeon were studied. The study followed the tenets of the Declaration of Helsinki, with approval from the local institutional review board. Informed consent was obtained from the subjects after explanation of the nature of the study. Patients with traumatic macular holes, high myopia

(>6 diopters), previous retinal surgery and previous ocriplasmin were excluded from the study. Only one eye per patient was entered into the study. All patients underwent transconjunctival 25 or 27 gauge vitrectomy with combined phacoemulsification and intraocular lens (IOL) implantation if phakic. Posterior hyaloid face separation was achieved with aspiration as required.

BBG-P was used to stain the macula in the first 35 cases and AV17-M in the second 30 cases. The technique of staining was to aspirate approximately 0.2 millilitres of the undiluted dye into the vitrectomy probe and then reflux the dye over the macula via the vitrectomy probe, using the proportional reflux function of the vitrectomy machine (Constellation, Alcon, USA). Due to the heavier than water density of the dye solutions, they both sink to the macular retina. After 10 seconds the dye was removed with aspiration, again using the vitrectomy probe, until the vitreous cavity was clear. A macular contact lens was used to view the peeling procedure. The ILM was peeled using a pinch technique and Grieshaber DSP 25g or 27g end gripping forceps and a peel radius of approximately 1 disc diameter.

At the end of surgery the surgeon made a subject score of the ease of tissue peeling based on the rigidity of the ILM using a visual analogue scale on a scale of 1-10 with 10 representing easily peeled ILM and 1 very difficult to peel tissue.

All surgeries were recorded via a beam splitter from the microscope (OPMI Lumera T, Carl Zeiss Meditec AG, Jena, Germany) for later analysis of the staining characteristics using a digital recorder (Medlife Mind Stream, Carl Zeiss Meditec AG, Jena, Germany). In particular at the end of each peeling procedure care was taken to obtain a well centred clear sequence of the peeled area. As part of the recording system setup routine, exposure and calibration alignments were performed, with adjustment of the white balance of the recording system.

The video sequences were reviewed and a frame displaying a well centred good quality image of the peeled area was selected and saved. The image was imported into the image viewing software GIMP and the mean colour in four selected areas in peeled, and adjacent non peeled retina, was measured in terms of the RGB colour space (Figure 1). These values were in turn converted into CIELAB color space using free software (www.colormine.org). The mean values for all images using one dye were found for the stained and adjacent peeled areas of retina and the Euclidean distance between these values in CIELAB color space using the CIE1976 algorithm, which can be regarded as a direct measure of the extent of the observed contrast, was calculated using the same software. To avoid confounding effects of different lighting conditions, the Euclidean distance was calculated only over the chromaticity components a^* and b^*, neglecting the lightness L^*.

Either 25 % SF6 or 20% C2F6 gas was used as a tamponade agent and the patients instructed to position face down for at least 5 hours a day for 3 days. Post-operative best corrected visual acuity (BCVA) at 3 months was measured using a standard Snellen acuity chart and converted to the logarithm of the minimal angle of resolution (logMAR) scores for the purposes of statistical analysis.

The ILM from the patients was placed immediately in 2% glutaraldehyde in 0.1M sodium cacodylate buffer. The ILM was enrobed in low-melting point agarose (4%) to form a small block. After secondary fixation in 2% osmium tetroxide, the samples were dehydrated in graded acetone, embedded in epoxy resin and polymerised at 60^{o}C. Ultrathin sections (70nm) were taken at 2 levels through the block, stained with uranyl acetate and lead citrate and viewed on a Philips CM100 transmission electron microscope.

For estimation of the amount of cellular tissue on the retinal side on the ILM, images were taken at x7900 magnification from 14 randomly sampled areas of the ILM. To quantify the amount of debris on each surface of the ILM a technique using grid line intersections was used (Figure 2) as we have previously described [11]. A similar technique was used to randomly sample ILM thickness. The graders for the ILM were masked to the dye used.

Statistical analysis

Descriptive and statistical analysis was performed using Minitab 17 version 1.0. Patients demographic characteristics, pre- and post-operative variables and macular hole features are presented in terms of mean, standard deviation and range or percentage as appropriate. Two-sample non paired t-tests were used to compare continuous variables. Associations between non-continuous variables were analysed using the Fisher's exact probability. Statistical significance was considered with a p-value of 0.05 or less.

Results

The study period was from January 2013 to March 2015. During the study period, 65 eligible patients underwent surgery. Fourteen eyes were excluded because of incomplete follow up (3), bilateral surgery (3), samples lost or inadequate (6) and video failure (2) leaving 51 patients data analysed. There were 26 in the BBG-P group and 25 in the AV17-M group.

The baseline characteristics are shown in table 1. The groups were well matched.

The outcomes are summarised in table 2. All but one hole in the BBG-P group closed. Visual outcomes were similar between the groups. There was no difference in ILM thickness between the two groups. The amount of vitreous side tissue present on the ILM was equivalent but the amount of retinal side tissue was significantly less in the AV17-M group compared to the BBG-P group.

There was a significant difference in the CIELAB colour space Euclidean separation distance with higher contrast in the BBG-P group.

Subjectively the AV17-M dye behaved in a very similar way to BBG-P with rapid sinking to the macular area, but easy removal using aspiration. It appeared to be ILM specific and did not stain areas of epiretinal tissue as with BBG-P. The ILM was slightly, but not statistically significantly, easier to peel in the BBG-P group than the AV17-M group (Mean visual analogue score 7.2 (SD2.2) versus 6.2(SD2.1) respectively), p=0.10).

Discussion

AV17 ($C_{41}H_{44}N_3NaO_6S_2$), also known as Coomassie violet R 200, is a violet coloured dye chemically similar to BBG **(**$C_{47}H_{50}N_3NaO_7S_2$) and belonging to the arylmethane dye group [12]. The preparation of AV17 we used was mixed with 5% mannitol to give it a density of 1.016g/cm^3 compared to BBG-P at 1.01 g/cm^3. The osmolality of the AV17-M preparation have been validated at 319 mOsm/kg, and pH at physiological levels [12-14].

Both dyes were used successfully to stain and then peel the ILM with visual acuity outcomes which were very similar between the groups. BBG-P gave significantly higher contrast

staining than AV17-M but the plane of ILM separation appeared to be deeper in the BBG-M group with higher amounts of retinal side ILM tissue than the AV17-M group.

We used the difference in position in CIELAB colour space as a measure of chromaticity measurements for the intensity of staining. This technique has been used by other researchers and provides a measure of the contrast visible to the human eye during the operation [15-17]. A difference in colour between two areas of less than 2.3 is generally considered to be perceptually equivalent [18]. Using the same technique Henrich et al. showed that ICG gave higher contrast staining than BBG [17]. The difference between these dyes and between the two we studied is most likely related to the separation of the dye colours from the background reddish colour of the retina/retinal pigment epithelium. It can be seen in Figure 3 showing the CIE1936 colour space, on which CIELAB color space is based, that violet is closer to the approximate colour of a caucasian fundus than blue or green. We used the same xenon light source in all surgeries to avoid the chromaticity measurements being influenced by the irradiation emission spectrum of the light source. The peak emission wavelength of Xenon is ~460nm. The maximum absorption of AV17 is 545nm and that of BBG 595nm. Henrich et al. found there was no difference in contrast effect when comparing illumination with a mercury vapour light source to a xenon one using BBG but it is possible that a different light source may have given different results [19].

The difference in contrast we found could also be related to a reduced intensity of staining reaction by AV17 on the ILM i.e. less uptake of stain. Henrich et al. have shown that BBG mixed with deuterium as a dye gave higher contrast staining than BBG without any heavy agent added ('light BBG') [20]. Similarly Totan et al. showed that light BBG used under air to stain the ILM gave higher contrast than light BBG used in the normal infusion solution, and

presumably intensity of stain would explain these findings [21]. We standardised the technique of staining between the two dyes and importantly used the same contact time between the dyes and retina, before washout. Both dyes showed selective ILM staining at this duration with low affinity for epiretinal membrane (ERM) as previously described for BBG, [22] and allowing the presence of epiretinal tissue on the ILM to be detected by negative staining as we have previously described for BBG [11]. This is a useful tool during surgery, allowing areas of bare ILM to be identified, but we were also careful to avoid areas of negative staining when making the contrast measurements by using areas of maximum stain around the peeled area as others have done [23]. Importantly the two groups were well matched in terms of the amount of vitreous debris present on the ILM, which, if unequal between the two groups, could have confounded contrast measurements. It is possible that AV17 may require a longer time for optimum ILM staining than BBG although contact time was found to have a limited effect on staining intensity with BBG in previous studies [22].

There was less retinal side tissue on the AV17-M eyes than the BBG-P eyes. Other authors have also found differences between different dyes in this respect with greater Muller cell remnants attached to the peeled ILM if no stain is used compared to ICG and ICG compared to BBG [2-4]. The mechanism for this alteration in ILM separation plane from the retina is unknown. The surgeon in this study noted a subjective difference in the ease of peeling of the ILM, with BBG-P stained ILM seeming to be more rigid and easier to peel than the AV17-M stained ILM. Vital dyes are known to alter the rigidity of the ILM with changes on atomic force microscopy being found on both retinal and vitreous sides of the ILM with both BBG and ICG [24]. Indeed increased rigidity is one of the explanations given for the surgically

experienced feeling that ILM is easier to peel after staining [24]. In experiments with anterior lens capsule , a material of similar composition to the ILM albeit thicker, the degree of increased rigidity induced by the dye varies by the type of dye used, with a less pronounced effect being found with trypan blue than BBG [25]. Atomic force measurements have not been performed with AV17 but it is possible that it induces less increase in rigidity than BBG using the contact time used. This in turn might alter the ILM separation plane and provide an explanation for our findings. The mechanism for the increased rigidity is uncertain but may relate to collagen cross linking [24,25]. We therefore measured ILM thickness but found no significant difference between the two dyes.

Kenawy et al have previously shown that the cleavage plane of the ILM to the retina can be altered by the presence of epiretinal tissue with a deeper plane of separation (i.e. greater amounts of retinal side tissue on the peeled ILM) in cases with significant ERM [26]. The two groups in this study were well matched for the amount of vitreous side material present on the ILM. Furthermore we have previously investigated this effect specifically in macular hole cases and did not find any correlation between the extent of vitreous side tissue and retinal side tissue [11]. It is possible however that factors which we didn't record e.g. hole chronicity and ERM thickness could have affected, and potentially confounded, our results.

ICG has known photosensitizing properties and its resultant decomposition products after illumination are thought to result in inner retinal toxic reactions, and resultant larger amounts of retinal side ILM cellular debris after peeling [1,6]. Neither BBG nor AV17 are thought to have any similar properties. Toxicity studies with AV17 have shown acceptable profiles in rabbit eyes at doses up to 0.50mg/ml and in an ex vivo bovine retinal model using AV17 mixed with deuterium oxide at doses of 0.125mg/ml [9,10]. At higher doses in the bovine model however evidence of inner retinal toxicity was seen histologically and on

electrophysiology [9]. The preparation we used had a concentration of 1.5 mg/ml and was mixed with 5% mannitol. We observed no discernible toxicity and visual acuity results were similar to the widely used dye BBG. It has been suggested that mannitol has antioxidant properties and it is possible that its inclusion in the formulation adds some cell protectant properties to explain our findings [27]. Similarly Awad et al showed a possible protective effect of PEG on RPE cells when exposed to trypan blue [28]. Importantly we also used a significantly shorter contact time than Tura et al. and indeed they found toxicity was strongly related to exposure duration as observed with other dyes [29].

There are several weaknesses to this study. Patients were not randomised although the surgical technique was standardised and all tested potential confounders between the groups were well matched. The surgeon who undertook the surgery measured the contrast measurements on the video images and could have biased the areas chosen for testing but conversely was able to avoid areas of non staining due to the presence of ERM etc. The effect of more prolonged dye contact time on contrast and ILM cleavage plane was not assessed which needs further study. Other than visual acuity we did not perform any other functional measures of vision and the clinical significance of the lower degrees of retinal debris in the AV17-M group is uncertain.

In conclusion both stains were successfully used in all cases to peel ILM with outcomes comparable to the published literature. AV17-M resulted in less retinal debris than BBG-P suggesting an altered and potentially beneficial ILM cleavage plane from the retina but with lowered staining contrast than BBG-P.

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Ethical approval: All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Legends

Figure 1

Representative fundal photograph captured immediately after ILM peel completion demonstrating the area of ILM peeled (using BBG-P in this case). The image was imported into the image viewing software GIMP. Using this program four 5 pixel square areas were selected in areas of maximally stained ILM and compared with four adjacent areas of the same dimensions in the same image where the ILM had been peeled.

Figure 2

Randomly selected area of ILM specimen at x7900 magnification. To quantify the amount of debris on each surface of the ILM, a grid of lines (line length 2µm) was superimposed on each image (A). The number of intercepts between the grid line and retinal surface were counted. Another grid (line length 1µm) was then superimposed on each image and the number of intercepts between the grid lines and any retinal or vitreous side tissue were counted (B). The percentage of surface covered by cellular tissue was taken as the number of intercepts on tissue / (number of intercepts on the ILM surface x 2) * 100.

Figure 3

The CIE 1931 color space chromaticity diagram

From "CIE1931xy blank" by BenRG - File:CIExy1931.svg. Licensed under Public Domain via Wikimedia Commons.
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